

Protection Against Chemical-Induced Lung Injury by Inhibition of Pulmonary Cytochrome P-450

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Protection afforded by trialkyl phosphorothionates against the lung injury caused by trialkyl phosphorothiolates probably results from the inhibition by the P=S moiety of the thionates, of one or more pulmonary cytochrome P-450 isozymes. The aromatic hydrocarbons *p*-xylene and pseudocumene also protect against this injury and inhibit some P-450 isozymes, but by a different mechanism.

OOS-Trimethylphosphorothionate and *p*-xylene were compared as protective agents against the effect of OOS-trimethylphosphorothiolate and two other lung toxins ipomeanol and 1-nitronaphthalene that are known to be activated by cytochrome P-450. The effects of these protective compounds, *in vivo*, on pulmonary cytochrome P-450 activity were also determined. Both compounds inhibited pentoxyresorufin *O*-deethylase activity, but not ethoxyresorufin *O*-deethylase. The phosphorothionate was most effective against lung injury caused by the phosphorothiolates and 1-nitronaphthalene, whereas *p*-xylene was much more effective against ipomeanol.

β -Naphthoflavone, which induces pulmonary ethoxyresorufin *O*-deethylase activity, did not protect against phosphorothiolate or 1-nitronaphthalene injury, and it was only marginally effective in decreasing the toxicity of ipomeanol.

Introduction

OOS-trimethylphosphorothiolate (OOSMeO) and OSS-trimethylphosphorodithiolate (OSSMeO) have been found as impurities in certain organophosphorus pesticides (1-3). When dosed to rats, they cause lung enlargement involving early necrosis of alveolar Type I pneumocytes and the proliferation of alveolar Type II cells (4).

In contrast, sublethal doses of two other systemic lung toxins 1-nitronaphthalene (1-NN) and ipomeanol, selectively damage the nonciliated bronchiolar epithelial (Clara) cells (5-9). Slightly higher doses, particularly of 1-NN, also damage the adjacent ciliated cells (9). Neither of these compounds injure Type I pneumocytes and do not induce cell proliferation in the alveoli.

The phosphorothionates, e.g., OOS-trimethylphosphorodithionate (OOSMeS), are not lung-specific toxins. When dosed to rats at 1/50 LD₅₀ dose, 2 hr prior to a challenge dose of OOSMeO or OSSMeO, they prevent the lung damage normally caused by these toxins (10,11).

The aromatic hydrocarbon *p*-xylene can also prevent

this lung damage when given 24 hr before challenge with OSSMeO (10).

The trialkylphosphorothiolates, like 1-NN and ipomeanol, require activation by the cytochrome P-450 system before they become toxic to the lung (3,6,7,12). In this preliminary study we have investigated the protection afforded against each of these toxins by both aromatic hydrocarbons and OOSMeS. We have also examined the effect of these protective compounds on the oxidation of pentoxyresorufin and ethoxyresorufin. These substrates are specific for the isozymes of cytochrome P-450 that are selectively induced by either phenobarbitone or 3-methylcholanthrene.

Materials and Methods

Animals

Female Wistar-derived rats (LAC:P) weighing 170 to 200 g (8-10 weeks old) were used throughout the study. All rats were housed in an air-conditioned animal room at 20 to 22 °C with a relative humidity of 40 to 60%. Oral dosing was by esophageal intubation after overnight starvation. Rats were subsequently given free access to water and standard (41B) diet. All test compounds were dosed by the PO or IP route as solutions in arachis oil (Sigma Chemical Co. Ltd., Dorset, England). LD₅₀ doses were

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calculated by the method of Weil (13), normally using groups of four rats, and lung weights were determined as described previously (11). Lungs were fixed *in situ* via the trachea, and slices 1 mm thick were prepared for light and electron microscopy (14).

Chemicals

OOSMeS and OOSMeO were donated by F. C. Churchill and J. W. Miles (Centers for Disease Control, Atlanta, GA). OSSMeO was prepared by P. B. Farmer and I. Bird (Medical Research Council [MRC], Toxicology Unit, Surrey). These compounds were all analyzed by E. Bailey (MRC Toxicology Unit) and found to be > 99% pure.

Ipomeanol (> 99% pure) was donated by M. Boyd (National Cancer Institute, Bethesda, MD). Ethoxyresorufin and pentoxyresorufin were donated by A. G. Smith (MRC Toxicology Unit) (15). 1-NN (> 99% pure), β -naphthoflavone (97% pure), and *p*-xylene (> 99% pure) were purchased from Aldrich (Aldrich Chemical Co. Ltd., Dorset, England). Cytochrome *c* and NADPH (tetrasodium salt) were obtained from Sigma (Sigma Chemical Co. Ltd., Dorset, England) and pseudocumene (98% pure) from Beckman (Beckman Ltd., Fullerton, CA).

Enzyme Assays

For lung microsomal preparation, rats were killed by decapitation, and homogenates of lung were prepared (20% in 0.05 M Tris; 0.154 M KCl, pH 7.4) using an Ildo X-1020 homogenizer at 75% maximum revolutions for a period of 25 sec. After centrifugation at 4°C (10,000*g* for 15 min and 100,000*g* for 60 min) the microsomal pellet was resuspended in Tris-KCl buffer. All enzyme assays were carried out on the day of microsomal preparation.

The *O*-dealkylation of alkoxyresorufin was measured fluorimetrically at 37°C by the direct monitoring of resorufin formation as described by Lubet et al. (16). NADPH-cytochrome *c* reductase was determined as the initial reduction rate (3 min at 25°C) of cytochrome *c* at 550 nm (17). The protein content of the microsomal preparation was measured by a modification of Lowry's method (18).

Results and Discussion

The oral administration of both OSSMeO (25 mg/kg) and OOSMeO (60 mg/kg) caused a selective injury to Type I pneumocytes within 24 hr, followed by lung enlargement and mortality after 3 to 5 days. Most deaths occurred 1 to 2 days after ipomeanol (18 mg/kg IP) and 1 to 7 days after 1-NN (299 mg/kg/PO). Ipomeanol (15 and 18 mg/kg IP) and 1-NN (87 mg/kg PO) resulted in damage to the bronchiolar epithelium within 24 hr, which was apparent by both light and electron microscopy. Damage was not restricted to the Clara cells at these doses, and it also extended to the ciliated cells (Fig. 1A). Lower doses could be employed to minimize damage to the ciliated cells, but the threshold for injury between ciliated and

nonciliated cells, particularly for 1-NN, was very fine. Ipomeanol and 1-NN also induced slight alveolar edema that was only detectable by electron microscopy. A few capillary endothelial cells were slightly swollen 24 hr after both compounds, but no changes were detected in any cells of the alveolar epithelium.

Most of the compounds used to inhibit cytochrome P-450 isozyme activity also provided some degree of protection against the toxins under investigation (Table 1). Protection by OOSMeS can be shown both by the 7-fold reduction in the LD₅₀ of OOSMeO and by the prevention of the increase in lung weights normally observed at 3 days (Table 2). Two hours after dosing with OOSMeS, the activity of lung microsomal pentoxyresorufin *O*-deethylase activity (PROD) was reduced by 90%. No change in the activity of ethoxyresorufin *O*-deethylase (EROD) was evident at this time, and neither *p*-xylene nor OOSMeS had any effect on cytochrome reductase levels (Table 3).

p-Xylene (at 24 hr after dosing) inhibited PROD, though slightly less effectively than OOSMeS. *p*-Xylene, when given 24 hr prior to challenge with OOSMeO, also gave a 5-fold protection against the lung toxicity, i.e., it was not quite as effective a protective agent as OOSMeS (Table 1).

This simple relationship between the inhibition of PROD and protection does not, however, hold for other toxins. OOSMeS only gave limited protection against ipomeanol, decreasing toxicity by approximately 2-fold; whereas *p*-xylene decreased the toxicity of ipomeanol 8-fold (Table 1). *p*-Xylene prevented all signs of bronchiolar and alveolar injury 1, 2, and 3 days after ipomeanol (18 mg/kg). Rats given *p*-xylene, followed by a much higher dose of ipomeanol (158 mg/kg), became moribund 3 days later. The bronchiolar epithelium showed no signs of injury or recent repair at this time (Fig. 1B), but macroscopic examination suggested that the liver had become the target organ. Pretreatment with OOSMeS reduced both the oral and intraperitoneal toxicity of 1-NN by a factor of four (Table 1). Pseudocumene, an aromatic hydrocarbon with properties similar to *p*-xylene, was only able to reduce the toxicity of 1-NN by a factor of two.

The trialkyl phosphorothiolates 1-NN and ipomeanol are all systemic lung toxins that are thought to require activation before they can produce toxicity. There is no evidence to suggest the hepatic activation of any of these compounds with subsequent export of toxic metabolites to the lungs, and so the lung itself is considered to be the activating organ.

A number of phosphorothionates that have been shown to prevent phosphorothiolate-induced lung injury also inhibit 7-ethoxycoumarin *O*-deethylase activity (7-ECOD), particularly in the lung (11). Several aromatic hydrocarbons including *p*-xylene and pseudocumene reduce the concentration of cytochrome P-450 in rat lung and also inhibit 7-ECOD (19). The activity of 7-ECOD has been characterized in rat liver (20,21) and reflects the net effect of several distinct isoenzymes. Despite this lack

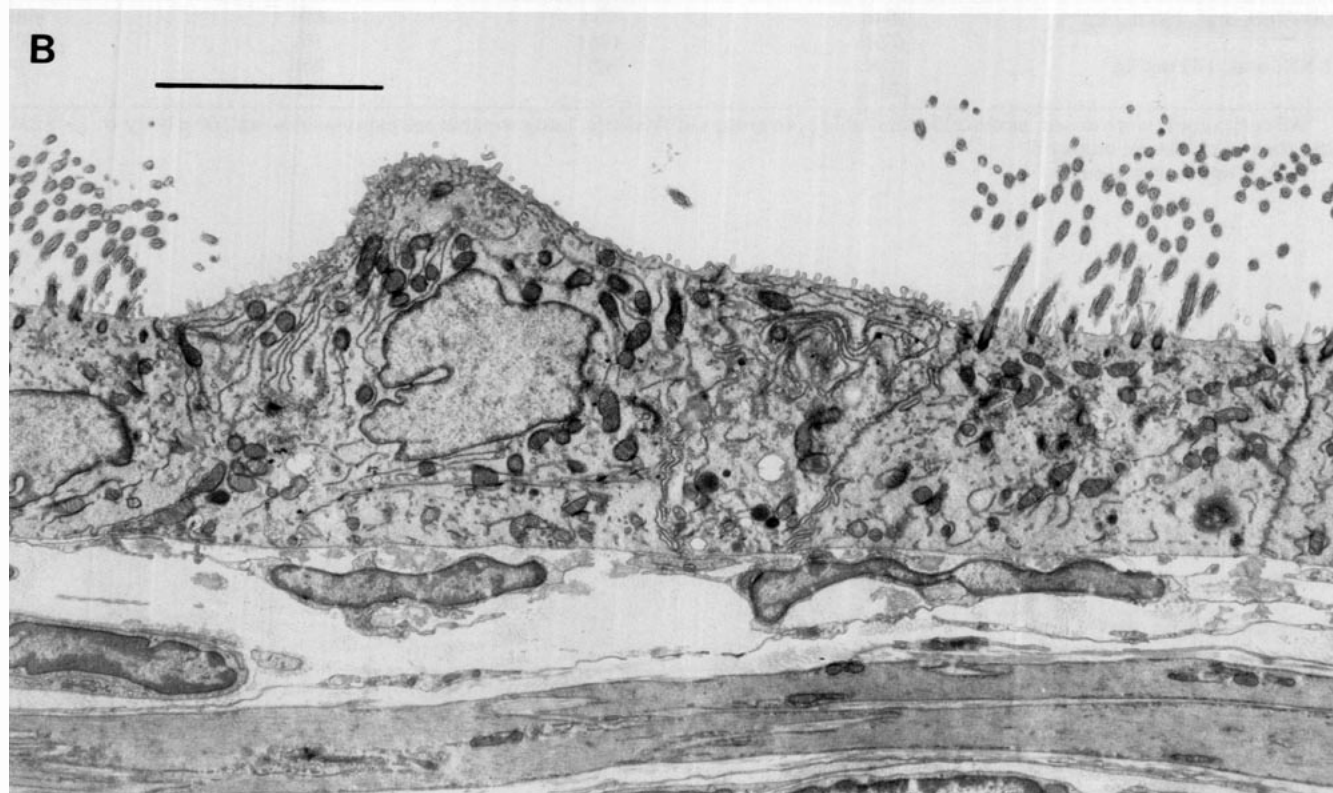


FIGURE 1. Rat bronchiolar epithelium: (A) 24 hr after the IP administration of ipomeanol (15 mg/kg) showing injury to both ciliated and nonciliated cells; (B) 72 hr after the IP administration of ipomeanol (158 mg/kg) to an animal previously dosed with *p*-xylene (1000 mg/kg). Bars = 5 μ m.

Table 1. LD₅₀ of toxins after various protective agents.^a

Protective agent/toxin	Aromatic hydrocarbons				
	Unprotected	<i>p</i> -Xylene, IP	Pseudocumene, IP	OOSMeS, oral	β -NF, oral
OOSMeO, oral	60 (51-71) ^c	343 (242-488)	ND ^b	417 (353-494)	54 (39-74)
Ipomeanol, IP	18 (14-22)	159 (133-190)	ND	40 (30-52)	45 ^d
1-NN, oral	299* (228-394)	ND	586 (477-719)	1067 (848-1343)	398 (279-568)
1-NN, IP	138 (106-178)	ND	ND	423 (298-602)	ND

^aEach group consisted of four rats, except *(10 rats). *p*-Xylene and pseudocumene (1000 mg/kg; 0.1 mL/100) were given 24 hr prior to challenge: OOS-trimethylphosphorothiolate (OOSMeS) (12.5 mg/kg; 0.2 mL/100 g) was given 2 hr before challenge, and β -naphthoflavone was given as three consecutive daily doses (3 \times 80 mg/kg 1.0 mL/100 g) before challenge on day 4. Unprotected animals were given the equivalent dose of arachis oil, the solvent used for all compounds.

^bND, not determined.

^cMilligram/kilogram body weight with 95% confidence limits.

^dEstimated LD₅₀ (45 mg/kg was maximum dose tested).

Table 2. Wet weight of lungs 3 days after the administration of each toxin to protected rats.^a

Protective agent/toxin	Unprotected	<i>p</i> -Xylene, IP	OOSMeS, oral	β -NF, oral
OOSMeO, oral, 100 mg/kg	1046 (55)	754 (54)	606 (5)	996 (8)
1-NN, oral, 173 mg/kg	928 (104)	ND ^b	568 (21)	ND

^aAll compounds were dosed, as described in Table 1, to groups of five rats. Lung weights are expressed as mg/100 g body wt (\pm SEM) at the time of challenge on day 0.

^bND = not determined.

Table 3. Enzyme activity of pulmonary microsomes (\pm SEM), prepared from rats given protective compounds and killed at the normal time for toxic challenge.^a

Protective compounds	Pentoxoresorufin <i>O</i> -deethylase	Ethoxoresorufin <i>O</i> -deethylase	Cytochrome <i>c</i> -reductase
Arachis oil	21.4 (1.5)	1.9 (0.28)	22.0 (0.92)
OOSMeS, oral, 2hr	1.7 (0.21)	1.9 (0.2)	16.3 (0.94)
<i>p</i> -Xylene, IP, 24 hr	2.8 (0.6)	ND ^b	23.4 (1.6)
Pseudocumene, IP, 24 hr	2.7 (0.28)	2.7 (0.16)	ND
β -Naphthoflavone, oral, 4 days	16.4 (1.6)	27.6 (2.3)	ND

^aPentoxoresorufin and ethoxoresorufin are expressed as pmole/min/mg protein and cytochrome *c* reductase as nmole min/mg protein. Compounds were dosed, as described in Table 1, to groups of five rats.

^bND = not determined.

of specificity, 7-ECOD is a good, overall indicator of P-450 content in rat lung (22). Three P-450 isozymes have, so far, been identified in rat lung (23), cytochromes P-450*b* and *e*, inducible by phenobarbitone; and P-450*c*, inducible by polycyclic aromatic hydrocarbons (23).

Pentoxylresorufin and ethoxylresorufin are much more specific substrates than 7-ethoxycoumarin (21). Pentoxylresorufin is particularly selective for P-450*b* (P-450 IIB1) (24), which is induced in liver by phenobarbitone (21). Ethoxylresorufin is specific for P-450*c* (P-450 IA), which is induced in liver by a number of polycyclic aromatic hydrocarbons.

The selective inhibition of lung, but not liver, cytochrome P-450 by *p*-xylene and pseudocumene is thought to result from the lack of alcohol dehydrogenase in lung (25,26). This organ, unlike the liver, is thus unable to detoxify the *p*-methylbenzyl alcohol formed during metabolism. *p*-Xylene may be metabolized to *p*-methylbenzyl alcohol in the lung by P-450IIB1 and probably also by other isozymes (26,27).

The alkylphosphorothionates probably inactivate cytochrome P-450 by oxidation of the P=S moiety and the subsequent covalent binding of atomic sulphur to cytochrome P-450 (11). This inactivation can also occur in the liver, but after an oral dose of OOSMeS (12.5 mg/kg), inhibition of PROD is greater in the lung (92%) than in the liver (50%) (Verschoyle and Dinsdale, unpublished data). The pulmonary activity of P-450IA1 assessed as EROD is very low, and it does not appear to be involved in the activation of these lung toxins. Some of the protective aromatic hydrocarbons actually induce EROD (19,22). β -Naphthoflavone, a potent inducer of EROD with no detectable effect on PROD (Table 3), gave no protection against the toxicity of 1-NN or OOSMeO (Table 1). β -Naphthoflavone only provided slight (2-fold) protection against ipomeanol toxicity, despite a 10-fold induction of EROD in lung and probably a much greater induction in liver. This suggests that either a reduced dose of ipomeanol is reaching the lung, owing to enhanced liver metabolism, or a very minor route of pulmonary detoxification has been induced. The induction of a fourth lung P-450 isozyme by β -naphthoflavone (27) may account for this minor route of metabolism.

This present study indicates that the protective effects of both the aromatic hydrocarbons and the trialkylphosphorothionates correlate, at least in part, with their inhibition of PROD in the lung. Cytochrome P-450IIB1 and other isozymes are obviously vital for the activation of each pneumotoxin, although their relative contributions are still being investigated.

The selective susceptibility of alveolar cells to OOSMeO and OSSMeO when injury by ipomeanol and 1-NN (which are similarly activated by the isozymes which comprise PROD) is largely restricted to the bronchiolar epithelium also remains to be explored.

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